## PROJECT DESCRIPTION

### Results of Prior NSF Support

The PI (DSH) has received three NSF research grants and one NSF equipment grant (listed below as grants 1-4). Grants 1 and 2 supported DSH from 1993 to 1999 as a postdoctoral fellow in the laboratory of Michael J. Donoghue (Harvard University). In September, 1999, DSH joined the faculty of Clark University. Grant 3, which began in September, 1999, and ends in August, 2002, has supported the Co-PI (MB) as a postdoctoral fellow in the laboratory of DSH since January, 2000. Publications for the last five years (including four publications with undergraduate co-authors) are given below. The following section discusses progress on the current grant only. Additional descriptions of our recent findings are presented in the "Background information" and "Preliminary results" sections of the proposal.

The research goals of our current grant are to develop broad, detailed phylogenetic hypotheses of the homobasidiomycetes and to infer the evolution of ecological and morphological features, including wood decay chemistries, mycorrhizal symbioses, and fruiting body morphologies. Sampling goals, which had to be reduced from those in the original proposal due to budgetary constraints, included: 1) development of a 125-species dataset with each species represented by four rDNA regions (nuclear and mitochondrial large and small subunit rDNA, 3.8 kb per species); 2) construction of large (about 500 species) phylogenetic trees using simultaneous and "supertree" analyses of overlapping rDNA datasets; and, 3) construction of a 24-species dataset using protein-coding gene sequences.

As noted, DSH moved to Clark University and began setting up his laboratory in September, 1999, and MB did not join the laboratory until January, 2000. Nevertheless, we have made significant progress and we are on track to complete the objectives of the present grant by August, 2002. So far, we have accomplished the following: 1) We developed a dataset of four rDNA regions that presently includes 100 species; we expect to reach our 125-species goal by September, 2001. An analysis of higher-level relationships of homobasidiomycetes based on a four-region dataset of 93 species is now in review (36). 2) We completed a phylogenetic study of the corticioid genus, *Aleurodiscus*, based on nuc-lsu rDNA sequences, which is now in press (180). 3) We completed a study of the relationships of the marine basidiomycete, *Nia vibrissa*, which is also in press (35). 4) We are analyzing a large dataset of overlapping sets of sequences from four rDNA regions, which includes 500 species. This dataset includes 190 nuc-lsu, 45 nuc-ssu, 121 mt-lsu, and 63 mt-ssu sequences that we have generated since September, 1999, as well as another 781 sequences that we generated previously or downloaded from GenBank. We will present the first analyses of this dataset in August, 2001, at the annual meeting of the Mycological Society of America. 5) We have amplified and sequenced a 600 bp region of cytochrome oxidase 3 from 21 species, using primers reported by Kretzer et al (107). However, preliminary analyses suggest that this gene will only provide resolution at low taxonomic levels. This fall we will begin investigating additional protein-coding genes that may help resolve deeper nodes.

In addition to supporting a postdoctoral fellow, the current grant has supported training and human resources development in the following ways: 1) Two Clark University undergraduates, Amanda Little and Brooke Barbera, have been supported on REU supplements, awarded in 2000 and 2001. Ms Little graduated in May, 2001, and is now employed as a research assistant at the Dana Farber Cancer Institute. 2) An African-American high school student, Damian Ramsey, from the Clark-affiliated University Park Campus School, will begin working in our lab in July and will continue during the academic year, 2001-2002, with support from a RAMHSS (Research Assistantships for Minority High School Students) supplement, awarded in 2001. 3) In April-August 2000, we hosted a visiting researcher, Dr. Sheng-Hua Wu, from the National Museum of Natural History, Taichung, Taiwan, who received training in fungal molecular systematics. 4) The presence of NSF support in our laboratory helped us obtain additional funding for an automated DNA sequencer (purchased with funds donated by a Clark University alumnus) and a microscopic image analysis facility (purchased with funds from grant 4, below), which have been used to train numerous undergraduates and graduate students.

**NSF Grants received:**

1. 1993: Postdoctoral Fellowship in Environmental Biology. Title: Molecular systematics of Polyporaceae. DEB-930268. $69,600.
2. 1996: Systematics and Population Biology (M. Donoghue, PI, DSH, Co-Investigator). Title: Molecular systematics of homobasidiomycetes, emphasizing Polyporaceae and Corticiaceae. DEB-9629427. $200,000. REU Supplements: 1997, 1998, $5,000 per year.
3. 1999: Systematics and Population Biology (DSH, PI). Title: Morphological and ecological diversification in the homobasidiomycetes: a molecular phylogenetic analysis. DEB-9903835. $190,000. REU supplements: 2000, 2001, $5,000 per year. RAMHSS supplement: 2001, $5000.
4. 2000: Multiple User Equipment Award in the Biological Sciences (D. Larochelle, PI; DSH, J. Thackeray, and T. Lyerla, Co-PIs). Title: Image analysis system for the Biology Department, Clark University. MUE-0070241. $42,454.

**Publications (since 1996; names of undergraduate co-authors are underlined):**

1. Hibbett, D. S. 1996. Phylogenetic evidence for horizontal transmission of Group 1 introns in the nuclear ribosomal DNA of mushroom-forming fungi. *Molecular Biology and Evolution* 13: 903-917.
2. Hibbett, D. S., and M. J. Donoghue. 1996. Implications of phylogenetic studies for conservation of genetic diversity in shiitake mushrooms. *Conservation Biology* 10: 1321-1327.
3. Hibbett, D. S., D. Grimaldi, and M. J. Donoghue. 1997. Fossil mushrooms from Cretaceous and Miocene ambers and the evolution of homobasidiomycetes. *American Journal of Botany* 84: 981-991.
4. Hibbett, D. S., M. J. Donoghue, and P. B. Tomlinson. 1997. Is *Phellinites digiustoi* the oldest homobasidiomycete? *American Journal of Botany* 84: 1005-1011.
5. Hibbett, D. S., E. M. Pine, E. Langer, G. Langer, and M. J. Donoghue. 1997. Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. *Proceedings of the National Academy of Sciences, U.S.A.* 94: 12002-12006.
6. Hibbett, D. S., and M. J. Donoghue. 1998. Integrating phylogenetic analysis and classification in fungi. *Mycologia* 90:347-356.
7. Hibbett, D. S., K. Hansen, and M. J. Donoghue. 1998. Phylogeny and biogeography of *Lentinula* inferred from an expanded rDNA dataset. *Mycological Research* 102: 1041-1049.
8. Hansen, K., D. S. Hibbett, and D. H. Pfister. 1999. Phylogenetic relationships of *Phillipsia* inferred from ribosomal DNA intergenic transcribed spacer sequences. *Mycologia* 91: 299-314.
9. Pine, E. M., D. S. Hibbett, and M. J. Donoghue. 1999. Evolutionary relationships of cantharelloid and clavarioid fungi. *Mycologia* 91: 944-963.
10. Hughey, B. D., G. C. Adams, T. D. Bruns, and D. S. Hibbett. 2000. Phylogeny of *Calostoma*, the gelatinous stalked puffball, based on nuclear and mitochondrial ribosomal DNA sequences. *Mycologia* 92: 94-104.
11. Hibbett, D. S., and R. G. Thorn. 2001. Basidiomycota: Homobasidiomycetes. Pp. 121-168 *in*: The Mycota, vol. VII part B, Systematics and Evolution (D. J. McLaughlin, E. G. McLaughlin, and P. A. Lemke, eds.). Springer Verlag.
12. Hibbett, D. S., Luz-Beatriz Gilbert, and Michael J. Donoghue. 2000. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* 407:506-508.
13. Taylor J., D. Jacobson, S. Kroken, T. Kasuga, D. Geiser, D. Hibbett, and M. Fisher. 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* 31:21-32.
14. Hibbett, D., and M. J. Donoghue. 2001. Analysis of correlations among wood decay mechanisms, mating systems, and substrate ranges in homobasidiomycetes. *Systematic Biology*. 50: 215-242.
15. Hibbett, D. S. 2001. Shiitake mushrooms and molecular clocks: Historical biogeography of *Lentinula*. *The Journal of Biogeography*. In press (accepted).
16. Binder, M., D. S. Hibbett, and H.-P. Molitoris. 2001. Phylogenetic relationships of the marine gasteromycete *Nia vibrissa*. *Mycologia*. 93(4):679-688.
17. Wu, S.-H., D. S. Hibbett, and M. Binder. 2001. Phylogenetic relationships of *Aleurodiscus* sensu lato and allied genera. *Mycologia*. 93(4):720-731.
18. Binder, M., and D. S. Hibbett. Higher level phylogenetic relationships of homobasidiomycetes inferred from four rDNA regions. *Molecular Phylogenetics and Evolution*. In review (submitted).

## PROPOSED RESEARCH

### Introduction

We propose to investigate the phylogenetic relationships of cyphelloid and aquatic homobasidiomycetes (mushroom forming fungi, ca 13,500 spp.; 79). There are about 300 species of cyphelloid homobasidiomycetes, which are characterized by minute, cup-shaped or tubular fruiting bodies that are often inverted (Fig. 1a; 8, 21, 47, 59-61, 162). Cyphelloid homobasidiomycetes have been nominally grouped in the family Cyphellaceae (or Porotheleaceae; 45, 47, 111, 128), but this group is widely regarded as artificial (21, 22, 58-60, 62, 162). Various authors have suggested that certain groups of cyphelloid homobasidiomycetes are related to agarics (mushrooms with a cap and gills), corticioid fungi (resupinate forms with smooth hymenophores), and polypores (10, 37, 47, 59, 71, 90, 152, 161-163). However, there is still no consensus regarding the phylogenetic distribution of cyphelloid homobasidiomycetes.

Aquatic (marine and freshwater) homobasidiomycetes include 11 meiosporic (sexual) species in 8 genera and about ten mitosporic (asexual) species in seven genera (92, 104, 119-122, 131, 133). Early-diverging clades of fungi include aquatic taxa with flagellated life stages (chytridiomycetes), but the plesiomorphic habitat of the basidiomycetes, which lack flagella, was probably terrestrial (29, 142). Therefore, marine and freshwater homobasidiomycetes probably represent secondary reversals to an aquatic habitat. Dramatic morphological changes have often been associated with such transitions, including the loss of ballistospory (forcible spore discharge), and the evolution of modified spores with elongate processes that presumably function in dispersal or attachment (Fig. 1b; 95, 96, 104). Consequently, it has been difficult to identify the closest terrestrial relatives of many aquatic homobasidiomycetes.

Recently, we discovered that the marine homobasidiomycete, *Nia vibrissa* (Fig. 1b), is closely related to the terrestrial cyphelloid fungus, *Henningsomyces candidus* (Fig. 1a; Binder et al., in press). Our results also suggested that the most likely candidate for the sister group of the *Nia-Henningsomyces* clade is a group that includes the terrestrial taxa, *Schizophyllum commune*, which has an unusual hymenophore composed of longitudinally split "gills" that curl inwards when dry (1, 66, 112, 134), and *Fistulina hepatica*, which has a hymenophore that appears to be poroid, but is actually composed of individual free tubes (114). Based on previous taxonomy (7, 21, 149) and recent molecular studies (108, 126), we infer that many (but not all) other cyphelloid species and several additional aquatic taxa are in the *Nia-Henningsomyces* clade and the *Schizophyllum-Fistulina* clade, along with certain resupinate and poroid taxa (see the "Background information" and "Preliminary results" sections). If this is correct, then these groups provide dramatic examples of morphological and ecological diversification in homobasidiomycetes.

The central goal of the proposed research is to delimit the major groups of cyphelloid and aquatic homobasidiomycetes, and identify their closest relatives. A particular focus will be to assess the relationships of the *Nia-Henningsomyces* clade and the *Schizophyllum-Fistulina* clade. This project will take advantage of the emerging database of homobasidiomycete ribosomal DNA (rDNA) sequences. Phylogenetic trees will be inferred using parsimony and maximum likelihood methods, and ancestral state reconstruction will be performed to estimate the pattern of transformations between cyphelloid and non-cyphelloid forms, as well as transitions between terrestrial and aquatic habitats. This project will use herbarium material and isolates from culture collections, as well as our own collections from New England, Panama, and Puerto Rico.

Training and outreach are important components of the proposed research. This project will continue the collaboration of DSH and MB, and will also support a Clark University graduate student (Zheng Wang) and undergraduate assistants (who we hope to support through REU supplements). In addition, this project will support a two-month research visit from a German graduate student, Ms Philomena Bodensteiner, who is studying taxonomy of cyphelloid fungi with Prof. Dr. R. Agerer. Finally, in collaboration with the Clark-affiliated University Park Campus School, this project will provide research opportunities for local high school students and high school biology teachers, who will receive professional development credits for research experience.

## Background information

**Taxonomy of cyphelloid fungi:** Cyphelloid homobasidiomycetes have a complex taxonomic history, a full account of which is beyond the scope of this proposal (2-23, 45-47, 49, 58-61, 101, 128, 140, 148-150, 159-161, 168, 169). Cooke (45, 47) included the majority of the cyphelloid homobasidiomycetes in the family, Porotheleaceae, which he suggested is a monophyletic group that was derived from corticioid ancestors. However, almost all other workers have viewed the group as a polyphyletic assemblage, based on differences in spore morphology, fruiting body ontogeny, hyphal anatomy, and other characters (21, 60, 61, 162). Most recent studies in this assemblage have been focused on defining individual genera (or clusters of genera) of cyphelloid fungi and identifying their closest non-cyphelloid relatives (e.g., 8, 18, 19, 21, 22, 159, 161).

We use the treatment of Donk (59) as the basis of our working classification of cyphelloid fungi. Donk recognized 26 genera, which he grouped solely for convenience in the "artificial family" Cyphellaceae. Later, Donk (60) and other workers erected additional cyphelloid genera, including *Calathella* (149), *Rectipilus* (7), and others. At present, there are approximately 34 recognized genera of cyphelloid homobasidiomycetes, with roughly 300 species. However, there is no recent conspectus of cyphelloid fungi, which makes it difficult to estimate the number of known taxa.

The polyphyly of cyphelloid homobasidiomycetes is generally accepted, but their precise phylogenetic placements are poorly understood. Singer (161, 162) and others (10, 59, 90, 152) have suggested that various genera of cyphelloid homobasidiomycetes were independently derived from agarics by a process of reduction. In his treatment of the Agaricales, Singer (162) placed 24 genera of white-spored cyphelloid fungi in the Tricholomataceae and placed four dark-spored genera in the Crepidotaceae. Several groups of pileate or capitate homobasidiomycetes in the Tricholomataceae sensu Singer with smooth or poroid hymenophores (e.g., *Favolaschia, Physalacria, Hispidocalyptella, Anastrophella*) have been interpreted as possible intermediates between agarics and cyphelloid forms (10, 21, 22, 90, 106, 162).

Relationships have also been suggested to exist between certain cyphelloid forms and corticioid forms (47). Donk (61) suggested that the type (and only) species of *Cyphella* s. str., *C. digitalis*, is closely related to the cupulate-corticioid genus *Aleurodiscus*, although this view was rejected by Singer (162). Other corticioid or cupulate forms that have been associated with cyphelloid forms include *Cytidia* and *Auriculariopsis* (163).

Several authors have noted similarities between cyphelloid forms and the pileate taxa, *Schizophyllum* (with longitudinally split "gills") and *Fistulina* (with "pores" composed of individual free tubes). Donk (61) included the cyphelloid genera *Henningsomyces* and *Stromatoscypha* in the Schizophyllaceae (*Plicaturopsis*, which produces minute pilei, was also included). In addition, Stalpers (163, also see 130) suggested that *Auriculariopsis* is closely related to *Schizophyllum*. Singer (162) suggested that the pseudoporoid fruiting body of *Fistulina* could have been produced by an aggregation of cyphelloid fruiting bodies on an expanded subiculum. *Fistulina* has been placed either in the Cyphellaceae, or in its own family, Fistulinaceae, in the suborder Cyphellineae (37, 47, 114). However, Donk (59, 61) and Agerer (10) rejected the view that *Fistulina* is closely related to cyphelloid forms. Finally, the minute, pendent polypore, *Porodisculus pendulus*, is noteworthy because it was originally classified as a *Cyphella* (*C. pendula* Fr.). Ginns (74) suggested that *Porodisculus* is closely related to the agaric, *Panellus*, and Gilbertson and Ryvarden (71, p. 679) suggested that it may be related to "the pleurotoid agarics or perhaps cyphellaceous fungi."

**Diversity of aquatic homobasidiomycetes:** There are only 11 described species (in eight genera) of sexual, aquatic homobasidiomycetes (92, 104). Four species have retained ballistospory and can be tentatively assigned to certain terrestrial groups based on morphology, but the rest are statismosporic (meaning that they have lost ballistospory; Fig. 1b) and have no obvious close relatives among terrestrial groups. Besides the sexual taxa, there are about ten species of mitosporic aquatic homobasidiomycetes, in seven genera.

***Ballistosporic aquatic homobasidiomycetes:*** *Halocyphina villosa* (Fig. 1a) and *Calathella mangrovii* are cyphelloid species that occur in intertidal mangrove habitats. *Halocyphina villosa* resembles the terrestrial cyphelloid genera *Henningsomyces* and *Rectipilus* (73), whereas *Calathella*

*mangrovii* is the only aquatic member of *Calathella*, which has eight species (21, 99). *Physalacria maipoensis* is the only aquatic species of *Physalacria,* which is a group of minute capitate species that Singer (162) classified among the "reduced series" of the Tricholomataceae (94). *Physalacria maipoensis* is found in mangroves, but it also occurs in adjacent forests that are not inundated, and is therefore regarded as "halotolerant" (94). Finally, *Gloiocephala aquatica* is the only aquatic species of *Gloiocephala*, which is another "reduced" genus of the Tricholomataceae (53, 162). *Gloiocephala aquatica* occurs fully submerged in freshwater, on *Scirpus* culms (53).

***Statismosporic aquatic homobasidiomycetes:*** This group includes seven species in four genera. *Nia* includes three marine species (*N. vibrissa, N. globospora, N. epidermoidea*) that produce minute, gasteroid (enclosed) fruiting bodies, and have appendaged basidiospores (Fig. 1b; 39, 55-57, 98, 104, 127, 176). *Nia* spp. occur on lignocellulosic substrates, and have also been recorded on feather and horsehair baits (28, 50, 77, 104, 109, 147, 153). *Digitatispora marina* and *D. lignicola* also occur in marine habitats, have appendaged basidiospores (Fig. 1b), and occur on wood, but they differ from *Nia* in having resupinate fruiting bodies (the morphology of the spores is also different; Fig. 1b; 54, 95, 100, 104). *Mycaureola dilseae* is a parasite of the marine red alga, *Dilsea carnosa*, that produces elongate, sigmoid basidiospores in minute, gasteroid fruiting bodies (118, 143). Finally, *Limnoperdon incarnatum* is a freshwater species that produces obovate spores within a gasteroid fruiting body, which Escobar and McCabe suggested resembles a cyphelloid form (64, 65). Nakagiri and Ito (129) compared fruiting body ontogeny in *Limnoperdon* and *Halocyphina* and found that both have a cyphelloid form at some point in development. However, *Limnoperdon* is cyphelloid early in ontogeny and later becomes gasteroid, whereas *Halocyphina* is initially gasteroid and only becomes cyphelloid at maturity (Fig. 1a).

***Mitosporic aquatic homobasidiomycetes:*** Genera of aquatic basidiomycetous hyphomycetes include *Arcispora, Dendrosporomyces, Fibulotaeniella, Ingoldiella, Taeniospora, Titaeella*, and *Tricladium*, all of which occur in freshwater (26, 52, 97, 119-122, 131-133, 157). In the few cases where the corresponding meiosporic forms are known, they appear to be related to certain terrestrial corticioid fungi: *Ingoldiella hamata* is the anamorph of *Sistotrema hamatum*; *Taeniospora descalsii* is the anamorph of *Fibulomyces crucelliger*; *Taeniospora gracilis* var. *enecta* is the anamorph of *Fibulomyces* sp. (or perhaps *Leptosporomyces galzinii*); and *Titaeella capnophila* is associated with *Hypnochus capnophilus* (26, 121, 131, 132). The mitosporic forms are often aeroaquatic, meaning that they produce aerial conidia from submerged substrates, and they also occur in water films on leaves and leaf litter, leading to the confusing term "terrestrial aquatic fungi" (27, 157).

**Molecular systematics:** Understanding of homobasidiomycete phylogeny has advanced dramatically since the early 1990s through the application of molecular techniques (42, 87). Most phylogenetic studies in homobasidiomycetes have used sequences of nuclear and mitochondrial large and small subunit ribosomal DNA (nuc/mt, lsu/ssu rDNA) or nuclear rDNA internal transcribed spacers (ITS), although a few protein-coding regions have also been used as sources of molecular characters (41, 42, 107, 171, 179). Typically, nearly full-length (1.8 kb) nuc-ssu rDNA sequences have been obtained, but the other three coding rDNAs have usually been represented by partial sequences (nuc-lsu, 1.1 kb; mt-ssu, 0.5 kb; mt-lsu, 0.4 kb).

Some of the largest, most taxonomically comprehensive homobasidiomycete molecular datasets include those of Bruns et al (154 spp./mt-lsu rDNA; 40), Langer (220 spp./nuc-lsu rDNA; 108), Hibbett et al. (161 spp./nuc-ssu, nuc-lsu, mt-ssu rDNA; 81, 82), and Moncalvo et al. (154 spp./nuc-lsu rDNA; 125). The dataset of Moncalvo et al. has recently been extended to over 800 species (Moncalvo and Vilgalys, unpublished; 126), including several cyphelloid taxa, as well as agaricoid taxa with putative cyphelloid relatives (discussed below). In addition to the studies just mentioned, there have been numerous studies focused on individual groups, including Boletales (31-34, 38, 78), Russulales (123), Gomphales (91), Hymenochaetales (175), Pleurotaceae (172), Amanitaceae (63), Cortinariaceae (113, 156), Coprinaceae (89), Polyporaceae (80, 103), and others. Summarizing the results of diverse molecular studies, Hibbett and Thorn (86) proposed a preliminary phylogenetic outline of the homobasidiomycetes as a whole, which divided the group into eight mutually exclusive major clades, which were given informal names (e.g., euagarics clade, russuloid clade, polyporoid clade, etc). The

classification of Hibbett and Thorn includes 246 genera of homobasidiomycetes and provides the most comprehensive higher-level phylogenetic framework for the group so far.

Despite all the activity in homobasidiomycete molecular systematics, few sequences of cyphelloid fungi have been produced. The unpublished nuc-lsu rDNA dataset of Moncalvo et al (generously provided by J.-M. Moncalvo and R. Vilgalys; 126) includes four cyphelloid forms, *Calyptella capula, Stigmatolemma poriaeforme, Lachnella alboviolascens*, and *"Porotheleum" (=Stromatoscypha) fimbriatum*, as well as *Porodisculus pendulus, Fistulina hepatica, Schizophyllum commune*, and several genera of "poroid agarics" (*Favolaschia*, *Poromycena*, *Mycenoporella*), which were also studied by Collins (44). Moncalvo et al's analyses suggest that the cyphelloid fungi are polyphyletic and are scattered among the white-spored agarics, as suggested by Singer (1986) and others. According to Moncalvo et al's results, the placements of several cyphelloid species were resolved (with varying levels of bootstrap support): *Calyptella capula* is closely related to *Hemimycena ignobilis*; *Stigmatolemma poriaeforme* is nested within *Resupinatus*; and *Stromatoscypha fimbriatum* is related to *Hydropus* sp. In addition, a clade containing *Schizophyllum commune*, *Fistulina hepatica*, and *Porodisculus pendulus* was resolved (with 54% bootstrap support). Langer's (108) nuc-lsu rDNA dataset included six cyphelloid species, *Henningsomyces candidus*, *Lachnella villosa*, *Cyphellopsis anomala*, *Cyphellopsis* sp., *Calyptella campanula*, and *Rectipilus fasciculatus*. As in Moncalvo et al's results, the cyphelloid forms are polyphyletic. One noteworthy (but weakly supported) clade resolved by Langer (108) includes *Schizophyllum commune*, *Dendrothele acerina, Cyphellopsis anomala*, and *Lachnella villosa*.

Several other studies have resolved placements of groups that are thought to be related to certain taxa of cyphelloid fungi. Nakasone's (130) molecular studies of the resupinate-discoid species *Auriculariopsis ampla* and *A. albomellea* suggested that *A. ampla* is closely related to *Schizophyllum commune*, as had been suggested (163), but that *A. albomellea* is closely related to *Phlebia*, which is in the polyporoid clade (85, 86). Therefore, Nakasone transferred *A. ampla* to *Schizophyllum* and *A. albomellea* to *Phlebia*. Finally, the cupulate-discoid genus, *Aleurodiscus* (which is putatively related to *Cyphella digitalis*, according to Donk [59] and others [110]), has been suggested to be a member of the russuloid clade (86, 180).

Only one aquatic homobasidiomycete has been included in molecular studies. As noted previously, our recent analyses of four rDNA regions strongly suggest that the marine gasteromycete, *Nia vibrissa*, is closely related to the terrestrial cyphelloid fungus, *Henningsomyces candidus* (35). Our analyses also suggest that *Fistulina hepatica* and *Schizophyllum commune* are closely related, and that the *Nia-Henningsomyces* clade is the sister group of the *Schizophyllum-Fistulina* clade. Freshwater mitosporic homobasidiomycetes have yet to be investigated using molecular approaches. However, three species of *Sistotrema*, which includes the putative teleomorph of *Ingoldiella hamata* (132) have been studied, and it appears to be polyphyletic, with species in the cantharelloid clade and the polyporoid clade (81).

**Summary—state of knowledge:** Cyphelloid and aquatic homobasidiomycetes make up a polyphyletic group of fungi that have putative relatives among diverse taxa of agarics, corticioid fungi, and polypores. Based on morphology, some ballistosporic aquatic homobasidiomycetes can be linked to terrestrial relatives, including the cyphelloid genera, *Calathella, Henningsomyces*, and *Rectipilus* (73, 99). However, the aquatic forms that have lost ballistospory are so morphologically divergent that their closest terrestrial relatives remain obscure.

To resolve the phylogenetic placements of cyphelloid and aquatic homobasidiomycetes, it will be necessary to evaluate them in the context of a taxonomically broad dataset of homobasidiomycetes. To a limited extent, this work has already begun. The studies of Moncalvo et al. (126) and Langer (108) have resolved several clusters of cyphelloid fungi. Our own work (35) provides strong support for two key clades, the *Schizophyllum-Fistulina* clade (also resolved by Moncalvo et al., 126) and the *Nia-Henningsomyces* clade, which appear to be sister taxa. Extrapolating from the studies of Nakasone (130), Moncalvo et al (126), and Langer (108), several other cyphelloid (*Lachnella, Cyphellopsis*), poroid (*Porodisculus*), and resupinate (*Auriculariopsis ampla*, *Dendrothele*) taxa can also be tentatively assigned to these groups. However, there has yet to be an analysis that includes all of these taxa simultaneously, and many relevant taxa have never been investigated.

## Research plan

The proposed research has three main goals: 1) Delimit the major clades of cyphelloid and aquatic homobasidiomycetes and identify their closest relatives; 2) Infer the historical pattern of transformations between cyphelloid and non-cyphelloid forms, as well as transitions between terrestrial and aquatic habitats; 3) Provide training in fungal systematics, and engage in outreach activities involving local high schools.

**GOAL 1: Delimit the major clades of cyphelloid and aquatic homobasidiomycetes and identify their closest non-cyphelloid and terrestrial relatives.**

**1. Taxon sampling, availability, and collecting:** We will generate sequence data from diverse cyphelloid and aquatic homobasidiomycetes and related taxa, and combine these data with existing datasets of homobasidiomycetes (see "Molecular regions for analysis", below). The heterobasidiomycete, *Auricularia auricula-judae*, will be used for rooting purposes, as suggested by several studies (165, 166). A tentative list of 108 target species is presented in Table 1. The list includes a broad sample of cyphelloid and aquatic homobasidiomycetes, with an emphasis on taxa that are potentially related to the *Schizophyllum-Fistulina* clade and the *Nia-Henningsomyces* clade, based on prior taxonomy and recent molecular phylogenies (see the "Background information" and "Preliminary results" sections; Figs. 2-3). These focal taxa include members of the Lachnellaceae and Cyphellopsidaceae sensu Agerer (22), as well as the genera *Henningsomyces*, *Calyptella*, *Physalacria*, and *Dendrothele*.

We will obtain sequences from cultures and herbarium materials. However, because of their small size, herbarium materials of cyphelloid fungi are not ideal for molecular studies. We will use only recent collections of taxa that are not available in culture. Potential sources of material include the herbaria of San Francisco State University, University of München, and others (see letters). Whenever possible, we will work from cultures. We have obtained 42 cultures from publicly accessible culture collections, including five species of aquatic homobasidiomycetes (Table 1), and we have requested cultures of six species of freshwater mitosporic homobasidiomycetes, which are deposited in the Centraalbureau voor Schimmelcultures and the Czech Collection of Microorganisms (Dr. L. Marvanová, personal communication). In addition, Dr. E. Langer (University of Tübingen) and Drs. J.-M. Moncalvo and R. Vilgalys (Duke University) have generously offered to provide DNAs of several cyphelloid species (see letters). Dr. E. B. Gareth Jones, an expert on marine fungi who is based in Thailand, has also offered to assist us by providing fresh collections as well as herbarium materials (see letter). Finally, Dr. W. Farnham (University of Portsmouth) has agreed to send herbarium material of *Mycaureola dilseae*, and Dr. L. Krieglsteiner (University of Regensburg) will send us collections of cyphelloid fungi from his ongoing collections in Germany. In total, DNAs, cultures, or recent collections are on hand or available for at least 58 species of cyphelloid and aquatic homobasidiomycetes and putatively related taxa (Table 1).

The materials that are on hand or readily available will permit a broad analysis of the phylogenetic relationships of cyphelloid and aquatic homobasidiomycetes. However, the value of this work will be significantly enhanced by the inclusion of additional species, and we would also like to study multiple accessions of certain critical or problematical taxa (e.g., *Henningsomyces candidus*). Therefore, we propose to undertake several collecting trips in the first year of the grant to collect cyphelloid and marine homobasidomycetes (however, we will not attempt to collect freshwater mitosporic basidiomycetes because we will receive cultures of these taxa). We will collect in New England, Panama, and Puerto Rico (see below). Published reports from the neotropics (17, 19, 20, 22, 47, 51, 93, 105, 148, 162) and eastern North America (43, 46, 72, 145, 149), and our own examination of the herbarium materials at DAOM (Ottawa) and FH (Massachusetts), suggest that about 75 of the 108 species listed in Table 1 may occur in the regions we will visit (for the rest, we will rely on cultures, herbarium materials, and donated specimens). However, we do not expect to obtain all of the species listed in Table 1. For planning purposes, we tentatively estimate that we will process about 100-150 new specimens (including cultures, herbarium materials, and field collections).

**TABLE 1. Target species**. Numbers in parentheses after generic names are estimated numbers of described species; numbers in parentheses after species names are numbers of individuals on hand or available (including DNAs, cultures, and recent herbarium materials). *Morphology, habitat, and anamorph-teleomorph relationships*: C=cyphelloid, A=aquatic, R=non-cyphelloid, but putatively related to cyphelloid fungi, M=mitosporic, aquatic basidiomycetes, T=putative, terrestrial teleomorphs of M. *Distribution*: TM=Temperate (North America and/or Europe), NT=neotropics, PT=paleotropics. *Sequence availability*: sequences generated in our lab are in bold type; sequences available elsewhere are in italics; 1=nuc-ssu, 2=nuc-lsu, 3=mt-ssu, 4=mt-lsu.

| **Arcispora (1)** | D. papillosa R, TM | **Ingoldiella (2)** | P. pendulus (1) R, TM,**2**,**3**,*2* |
|---|---|---|---|
| A. bisaggitaria (1) M, | **Digitatispora (2)** | I. hamata (1) M | **Phlebia (132)** |
| **Amyloflagellula (4)** | D. lignicola A, TM | **Lachnella (13)** | P. chrysocrea (1) R, TM,**3** |
| A. pseudoarachnoidea C,NT | D. marina A, TM | L. alboviolascens (2) C, TM,*2* | P. radiata (1) R, TM,**1**,**2**,**3**,**4** |
| A. inflata C, TM | **Episphaeria (1)** | L. tiliae C, TM | P. tremellosa (1) R, TM,**2**,**3** |
| **Auriculariopsis (2)** | E. fraxinicola C, TM | L. subfalcispora C,NT | **Rectipilus (7)** |
| A. albomellea (1) R, TM,**2**,**3**,**4**,*2* | **Favolaschia (58)** | L. villosa (2) C, TM,**2**,**3**,**4** | R. confertus C, TM |
| A. ampla (4) R, TM,**2**,**3** | F. calocera (71) C,PT | **Limnoperdon (1)** | R. fasciculatus (3) C, TM,*2* |
| **Calathella (8)** | F. pezizaeformis (4) C,PT | L. incarnatum (1) C/A,PT,**2**,**4** | R. sulfureus C, TM |
| C. mangrovii (1) C/A,PT,**2**,**3** | F. pezizoidea (1) C,NT,**2**,**3**,**4** | **Maireina (28)** | **Schizophyllum (8)** |
| **Calyptella (18)** | F. pustulosa (9) C, TM | M. ilicis C, TM | S. commune (2) R, TM,**1234** |
| C. campanula (1) C, TM,*1* | **Fibulomyces (5)** | M. jacksonii C, TM | S. fasciatum (1) R,NT,**2** |
| C. capula (1) C, TM,**1** | F. crucelliger T, TM | M. monacha C, TM | S. radiatum (1) R,NT,**2**,**3**,**4** |
| **Cellypha (1)** | F. mutabilis T, TM | **Merismodes (5)** | S. umbrinum (1) R,NT,*2* |
| C. goldbachii (1) C, TM | **Fibulotaeniella (1)** | M. fasciculatus (1) C, TM,**2**,**3** | **Seticyphella (3)** |
| **Chaetocalathus (11)** | F. canadensis (1) M, TM | M. ochraceus (1) C, TM | S. punctoidea C,NT |
| C. craterellus C,NT | **Fistulina (14)** | **Metulocyphella (2)** | S. tenuispora C, TM |
| C. carnelioruber C,NT | F. antarctica (2) R,NT,**2**,**3** | M. lanceolata C,NT | **Sistotrema (117)** |
| **Chromocyphella (3)** | F. hepatica (3) R, TM,**1**,**2**,**3** | M. rostrata C,NT | S. eximum (1) T, TM,**1**,**2**,**3**,**4** |
| C. crouanii C,NT | F. pallida (1) R, TM,**2**,**3** | **Mycaureola (2)** | S. hamata T, |
| C. muscicola (1) C,NT | **Flagelloscypha (33)** | M. dilseae TM | S. musicola (1) T, TM,**1**,**2**,**3**,**4** |
| **Cyphella (1)** | F. abieticola C, TM | **Nia (3)** | S. sernanderi (1) T, TM,**2**,**3** |
| C. digtalis (1) C, TM,**2**,**3** | F. minutissima (1) C, TM,**2** | N. epidermoidea R/A, TM | **Sphaerobasidioscypha (2)** |
| **Cyphellopsis (3)** | F. polylepidis C,NT | N. globospora R/A, TM | S. citrispora C,PT |
| C. anomala (3) C, TM,**2**,**3**,**4**,*2* | F. punctiformis C, TM | N. vibrissa (4)R/A,TM,NT,**1234** | S. oberwinkleri C,NT |
| C. subglobispora C,NT | **Gloiocephala (39)** | **Nochascypha (4)** | **Stigmatolemma (5)** |
| C. sp. (1) C, TM,*2* | G. aquatica (1) R,NT | N. filicina C, TM | S. poriaeforme (2) C, TM, **2**,*2* |
| **Cyphellostereum (1)** | G. epiphylla R, TM | N. stricta C,NT | S. taxi (1) C, TM |
| C. laeve (2) C, TM | G. menieri (1) R, TM,*2* | **Pellidiscus (2)** | **Stromatocyphella (2)** |
| **Cytidia (14)** | **Halocyphina (1)** | P. pallidus (1) R, TM | S. aceris C, TM |
| C. lanata R, TM | H. villosa (3) A,PT,**2**,**3**,**4** | P. pezizoidea R, TM | S. conglobata C, TM |
| C. salicina (2) R, TM,**2** | **Henningsomyces (14)** | **Physalacria (16)** | **Stromatoscypha (3)** |
| C. rutilans R, TM | H. candidus (2) C, TM,**1**,**2**,**3**,**4** | P. bambusae (1), R,PT,**2**,**3**,**4** | S. fimbriata (2) C, TM,**2**,*2* |
| **Deigloria (8)** | H. puber (1) C, TM | P. inflata (1) R, TM, **2**,**3**,**4** | **Taeniospora (3)** |
| D. pulchella C,NT | H. pulchellus C, TM | P. maipoensis (1) R,PT,**2** | T. gracilis (1) M |
| D. subpeltata C,NT | **Hormomitraria (3)** | P. aff. orinocensis (1) R,NT,*2* | T. nasifera (1) M |
| **Dendrosporomyces (1)** | H. albidula R,NT | P. sp. (1) R,PT | **Woldmaria (2)** |
| D. prolifer M, | H. sulfurea R, PT | **Plicaturopsis (2)** | W. crocea (1) C, TM |
| **Dendrothele (17)** | **Incrustocalyptella (2)** | P. crispa (1) R, TM,**1**,**2**,**3**,**4** | |
| D. acerina (1) R, TM,*2* | I. colombiana C,NT | P. scarlatina R,PT | |
| D. microspora R, TM | I. pseudopanacis C,NT | **Porodisculus (1)** | |

Collections will be divided in the field into two parts; one part will be used for culturing and morphological study, and the other will be placed in silica gel for DNA isolation. At the conclusion of the project, specimens will be deposited in the Farlow Herbarium of Harvard University (see letter from Dr. D. Pfister). Locations and a tentative schedule for fieldwork are described below:

***New England*:** New Hampshire, White Mountain National Forest; Massachusetts, Harvard Forest, Petersham, and other locations (September, 2002, July-August, 2003). These locations are within a one to three hour drive of our laboratory and provide access to deciduous and conifer-dominated forests.

***Puerto Rico***: Caribbean National Forest and Magayas Island Marine Biology Station (November 1-14, 2002). These locations provide access to upland rainforest and mangrove communities. We will base our collecting at the USDA Forest Products Laboratory in Luquillo, which is fully equipped for mycology (microscopes, laminar flow hood, etc). Dr. Jean Lodge (USDA Center for Forest Mycology Research, Puerto Rico) has agreed to help coordinate this collecting trip (see letter). We will not require a permit for collecting in the CNF, but we will request permits from the Department of Natural Resources and Environment for collecting in the mangroves.

***Panama*:** Smithsonian Tropical Research Institute (STRI), Bocas del Toro Research Station, Barro Colorado Nature Monument, Fortuna Research Station (June 1-30, 2003). These sites, which are all part of the STRI network of research facilities, provide access to lowland and montane rainforests (BCN, Fortuna) as well as mangrove communities (Bocas del Toro). STRI will facilitate processing of permits for collecting in Panama (see letter).

In addition to the trips described above we will travel to Friday Harbor, Washington, in September, 2001, with the specific aim of collecting *Digitatispora lignicola* (no NSF support is requested). We will be accompanied by Dr. Gareth Jones, who informs us that he has repeatedly collected *D. lignicola* at this site (see letter).

**2. Culturing and morphological studies:** We will attempt to culture all of the fungi that we collect. Cultures will be used for DNA isolation (supplementing field-collected fruiting bodies in silica gel), and will be deposited at DAOM (Ottawa) and FPL/USDA (Madison). Terrestrial fungi will be cultured by obtaining spore-drops onto malt-extract agar (MEA). For marine fungi, we will employ techniques developed by Schimpfhauser and Molitoris (155) and others (124, 129, 151, 174). We have used these methods to culture the marine fungi *Nia, Halocyphina*, and *Calathella* (see "Preliminary results", 35). We will attempt to generate fruiting bodies in culture for all isolates (of sexual species) that we receive from culture collections, and we will also seek voucher specimens for morphological study (see below). We will experiment with a variety of media for fruiting, including MEA, glucose-yeast-peptone agar, and sterilized sawdust-wheat bran. We have used these methods previously to fruit wood-decaying basidiomycetes (70, 83, 84).

We will undertake morphological studies of all the specimens used in molecular analyses (including our collections, voucher specimens of cultures, fruiting bodies produced in culture, etc). Sections of fruiting bodies will be made by hand or with a freezing microtome, and typically will be mounted in water, KOH, HCl, and Melzer's reagent. Morphology and staining reactions of spores, basidia, surface hairs, and context hyphae will be recorded using a Nikon E600 compound microscope with bright field, phase-contrast, and Nomarski optics, with a Spot RT-slider digital camera and dedicated Macintosh G4 computer. Occasionally, we will visit the Farlow Herbarium of Harvard University (about a one hour drive from Clark University) to compare our collections to those on deposit (including the Patouilliard herbarium), many of which have been annotated by relevant authorities, including Agerer, Cooke, and Singer (see letter from Dr. D. Pfister). We will also use the excellent library of taxonomic mycology at the Farlow. As necessary, we will request loans from other herbaria to confirm identifications.

**3. Molecular regions for analysis:** We propose to develop two datasets, one composed of nuc-lsu rDNA (about 1.1 kb), and another composed of four regions of rDNA, including the nuclear and mitochondrial small and large subunit rDNA (hereafter the "four-region" dataset, about 3.8 kb). We will generate nuc-lsu rDNA sequences for every isolate that we process, and add these sequences to a taxonomically broad "reference" dataset composed of published sequences. Following analyses of the reference nuc-lsu rDNA dataset, we will select exemplars of unique clades of cyphelloid and aquatic homobasidiomycetes to be included in the four-region dataset.

***Analyses of nuc-lsu rDNA:*** We selected the nuc-lsu rDNA region for the initial screening of cyphelloid and aquatic homobasidiomycetes for two reasons: 1) it is well characterized and has repeatedly been shown to provide resolution of terminal clades in homobasidiomycetes (32, 33, 63, 89, 91, 125, 126, 172, 175); and, 2) a large number of sequences of diverse homobasidiomycetes are already available in GenBank (see "Background information"). We will select 100-200 published sequences for the reference dataset, to which we expect to add about 100-150 sequences (see "Taxon sampling", above,

and Table 1). Thirty-four nuc-lsu rDNA sequences are available for the species listed in Table 1. Therefore, we will generate about 76-126 new nuc-lsu rDNA sequences.

As noted previously, several groups of cyphelloid homobasidiomycetes have been shown to be nested in the euagarics clade (which contains over half of all described homobasidiomycetes), so sampling in the reference dataset will emphasize this group (focusing on Tricholomataceae). Nevertheless, relationships have also been suggested to exist between certain cyphelloid and aquatic homobasidiomycetes and diverse taxa outside of the euagarics clade (see the "Background information" section). Therefore, it will be necessary to include representatives of all major groups of basidiomycetes in the reference nuc-lsu rDNA datasets. Based on the results of initial analyses, the taxonomic composition of the reference dataset may be reconfigured to allow more precise placement of cyphelloid and aquatic taxa.

***Analyses of the four-region dataset:*** Although the nuc-lsu rDNA provides strong support for many terminal groupings, it does not provide robust resolution of the major clades of homobasidiomycetes, as defined by Hibbett and Thorn (86). Recently, we performed analyses of a four-region dataset of 93 diverse homobasidiomycetes (36). The four-region dataset provided much stronger support for the major clades of homobasidiomycetes (except the polyporoid clade, which is weakly supported by all data partitions) than the nuc-lsu rDNA data alone. For example, the monophyly of the euagarics clade received less than 50% bootstrap support with nuc-lsu rDNA alone, whereas with the four-region dataset the euagarics clade was supported at 76-85% bootstrap support (the bootstrap values vary somewhat depending on taxon sampling; 36). Similarly, the "core euagarics clade" was weakly supported by the nuc-lsu rDNA alone (bootstrap <50%), but was strongly supported by the four-region dataset (bootstrap 78-98%). Therefore, to place the cyphelloid and aquatic homobasidiomycetes in a higher-level phylogenetic context (for example, to assess their inclusion in the euagarics clade), it will be necessary to include them in a multi-gene dataset that includes representatives of all major clades of homobasidiomycetes (sensu Hibbett and Thorn, 86). In our ongoing research (36), we have developed a taxonomically broad four-region dataset (currently 100 species), to which we expect to add about 50 species. Based on our estimates of sequence availability (Table 1), we will need to generate about 41 nuc-ssu, 24 mt-ssu, and 34 mt-lsu rDNA sequences (in addition to the nuc-lsu rDNA sequences).

**4. Molecular techniques:** Techniques of molecular systematics have become standard and therefore will not be described in detail. We will isolate DNA from mycelia and fruiting bodies using a SDS-NaCl extraction buffer, followed by organic extractions and ethanol-sodium acetate precipitation, or GeneClean (Bio101) purification. In most cases, we will amplify rDNAs using standard primers and protocols (135, 173, 179). However, for *Mycaureola dilseae* (the marine red algal pathogen) we will need to use fungal-specific primers to avoid amplification of algal rDNA. A large number of red algal rDNA sequences are available in GenBank (30), which should allow us to design fungal-specific primers. In most cases, we will sequence PCR products directly. However, for *M. dilseae* and others it may be necessary to clone the amplified rDNAs, which will be done using the pGEM T-easy vector kit (Promega) and gel-purified PCR products. Sequencing will be performed using ABI Prism fluorescent dye-terminator chemistry and an ABI 377XL automated DNA sequencer (Applied Biosystems). Sequences will be assembled using Sequencher (GeneCodes Corp.) and will be aligned with a combination of computer methods (e.g., Clustal) and manual adjustment.

**5. Phylogenetic analyses:** Phylogenetic analyses using parsimony and maximum likelihood will be performed using PAUP* 4.0 (167).

***Parsimony analyses:*** Our datasets will typically include 100-300 taxa, which will limit us to heuristic search methods. To improve our chances of finding the most parsimonious trees, we will use a two-step search protocol (117, 136): 1) up to 1000 heuristic searches will be performed, with random taxon addition sequences and TBR branch swapping, but keeping only two trees per replicate; 2) the set of shortest trees found in step 1 will be used as starting trees for TBR branch swapping, with MAXTREES unrestricted. Bootstrap analysis (69, 154) will be performed to estimate topological robustness. To run the bootstrap in a reasonable amount of time, it will be necessary to abbreviate the search protocol. Therefore, each replicate will use a single-step heuristic search, with one starting tree generated with a random taxon addition sequence, TBR branch swapping, and MAXTREES restricted.

Parsimony analyses will employ two different schemes for weighting nucleotide substitutions: 1) all transformations weighted equally; and, 2) differentially weighted transformations, with step matrix values based on substitution probabilites estimated using the "pairwise base differences" option in PAUP* or maximum likelihood estimates of transformation probabilities, as applied by Moncalvo et al. and Lutzoni et al. (115, 125). Differentially weighted parsimony has been suggested to improve phylogenetic accuracy relative to equally weighted parsimony (25, 48, 88). However, it increases run times significantly, which may require that we reduce the stringency of individual searches.

Most parsimony analyses will exclude hypervariable, ambiguously aligned regions. However, we will also explore the method of Lutzoni et al. (115), in which hypervariable regions are coded as single characters using stepmatrices, with each unique sequence coded as a single character state (for alternative methods of handling ambiguous regions, see 177, 178). One potential limitation of this method, is that in PAUP* a maximum of 32 character states are allowed, but there may be more than 32 unique sequences of some hypervariable regions in our dataset (115). Assuming that we can use Lutzoni et al.'s method, there will be four possible character coding regimes for parsimony, involving equally or differentially weighted nucleotide transformations, and inclusion or exclusion of hypervariable regions, which will allow us to assess the sensitivity of our results to variation in coding schemes.

***Maximum likelihood (ML) analyses:*** ML analyses are computationally intensive and will be difficult with the large datasets that we will generate. The following strategy is designed to expedite ML analyses: 1) Mininum-length trees from parsimony analyses will be used as starting trees for branch swapping with ML as the optimality criterion; 2) Prior to initiating branch swapping, the optimal model of evolution will be determined, using the program Modeltest (144), which performs a hierarchical set of likelihood ratio tests, designed to find the simplest (least parameterized) model that adequately explains the data; 3) Model parameters (e.g., nucleotide frequencies, substitution rates, number of rate categories, and gamma distribution shape parameter) will be estimated using PAUP*; 4) Model parameters will be fixed and branch swapping will be performed, using the most exhaustive swapping algorithm possible. We do not intend to perform bootstrap analysis using ML.

***Analysis of conflict among data partitions:*** The four-region dataset will contain sequences from the (unlinked) nuclear and mitochondrial genomes. Prior to combining these data, we will attempt to assess whether they have different underlying phylogenies (e.g, resulting from hybridization), which could be a source of error in combined analyses. Commonly used methods for assessing such conflicts include the Kishino-Hasegawa (102) likelihood ratio test and the Templeton (170) non-parametric test. However, the statistical validity of these tests has recently been questioned (76, 158). Alternative tests, reviewed by Goldman et al. (76), include the Shimodaira-Hasegawa (SH, 158) test and methods using the parametric bootstrap. Both are computationally intensive. For example, according to Goldman et al. (76, p. 660), the SH test requires comparison of all trees "that can possibly be entertained as the true topology", which is not feasible for datasets with large numbers of taxa. Another popular method for detecting conflict among data partitions is the ILD or partition-homogeneity test (67, 68). However, this method is limited because it does not identify the individual taxa involved in conflicts, and it can return a positive result when different data partitions have strongly different rates of evolution, yet have the same underlying phylogeny (48, 164). Thus, none of the currently available methods for assessing conflict among data partitions are ideal. We will perform independent bootstrapped parsimony analyses of the nuclear and mitochondrial data partitions and compare bootstrap consensus trees for evidence of strongly supported positive conflict (equated with bootstrap support of at least 90%). We will delete taxa involved in conflict, or delete certain molecular regions for individual taxa. For cases involving critical taxa, we will further explore conflicts using the SH test (with a sample of possible trees) and the parametric bootstrap (76, 158).

**GOAL 2: Infer the historical pattern of transformations between cyphelloid and non-cyphelloid forms, as well as transitions between aquatic and terrestrial habitats**.

Ancestral state reconstruction (ASR) will be performed to address the following questions:

- How many times have cyphelloid forms evolved?
- What are the morphological precursors of cyphelloid forms—are they all "reduced agarics"?

- Have cyphelloid forms given rise to other forms? Specifically, are *Schizophyllum, Fistulina, Porodicsulus*, and *Auriculariopsis ampla* derived from cyphelloid forms?
- How many times have homobasidiomycetes made the transition to aquatic habitats? Have there been any reversals from aquatic to terrestrial habitats?

ASR will be performed using parsimony (implemented in MacClade, 116) and ML methods (implemented in Discrete, 137-139) on trees derived from the nuc-lsu rDNA dataset as well as the four-region dataset. Using parsimony, we will experiment with binary character codings (e.g., cyphelloid/non-cyphelloid) and multi-state codings (e.g., cyphelloid/tubular/lamellate/smooth, etc). With binary character coding, we will vary the relative costs of losses vs. gains, which will provide a crude measure of the "robustness" of the inferred ancestral states (146).

ML-based ASR will use the "local" method of Pagel (139). Discrete only allows analysis of binary characters. A strength of the ML method, however, is that it incorporates branch length information in estimates of ancestral states. We will perform ML-based ASR with branch lengths estimated using maximum likelihood. Following Pagel (139) and others, a difference of two units of log likelihood will be taken to represent "strong" support for one character state assignment over another at a node.

**GOAL 3: Provide training in fungal molecular systematics and engage in outreach activities to local high schools.**

The proposed research will provide full-time support for a post-doctoral fellow (MB), who will have primary responsibility for all aspects of the research, and summer support for a Ph.D. student (Zheng Wang), who will assist with morphological and molecular studies and fieldwork. We will also provide a research experience for one Clark University undergraduate in each year of the project, who we hope to support through REU supplements, as we have done in the past. Finally, we will host a visiting graduate student, Ms Philomena Bodensteiner, for two-months (tentatively, Jan.-Feb., 2003). For her dissertation research, Ms Bodensteiner is studying anatomy of cyphelloid fungi under the direction of Dr. Reinhard Agerer (University of München), who is a world authority in the morphology and taxonomy of this group (2-24).

We will engage in outreach activities involving the University Park Campus School (UPCS), which is an ethnically diverse public high school that is affiliated with Clark University. We propose to provide training for one UPCS student for six weeks in the summer (Jul. 1-Aug. 15), in each year of the project. This schedule is designed to coordinate with the two-month UPCS summer vacation, and our own commitments to fieldwork and meetings. The high school student will receive training in techniques of molecular systematics (culturing, DNA isolation, PCR, DNA sequencing) and will be involved in local fieldwork. We also propose to provide similar training, during the same time periods, for a local high school biology teacher. This research experience will count toward professional development points, which are required for recertification of high school teachers by the Massachusetts Department of Education, and which Clark University is authorized to assign (see letter from M. Shepard). In the first year of the project we plan to host Ms Jody Bird, who is a biology teacher at UPCS and a Clark alumna (see letter). Later, we will solicit applications from throughout the Worcester public school system. The high school student and teacher will attend weekly lab meetings, and will be supervised jointly by DSH, MB, and ZW.

**Relation to long-term goals of the PI**

The long-term goals of the PI's research program are to produce comprehensive phylogenetic trees of homobasidiomycetes, and understand the evolution of important morphological and ecological features. In prior work, phylogenetic trees have been used to study evolution of major fruiting body forms (e.g., polypores, agarics, gasteromycetes; 80, 85) and nutritional modes (brown and white rot decay types, ectomycorrhizal symbioses; 81, 82), and a preliminary phylogenetic framework for the homobasidiomycetes has been developed (86). The proposed research is a logical extension of this work because it concerns the evolution of an enigmatic, morphologically-defined assemblage (cyphelloid forms) as well as ecological shifts between terrestrial and aquatic habitats. The training component of

the project serves additional long-term goals of the PI, which are to promote evolutionary biology and increase awareness of the diversity and importance of fungi.

**Relation to work in progress elsewhere**

The proposed research is most closely related to the ongoing studies of agarics and related homobasidiomycetes by Drs. J.-M. Moncalvo and R. Vilgalys (Duke University). Our proposed study will capitalize on the nuc-lsu rDNA database that is being developed in the Vilgalys lab (and elsewhere), and will complement their project by sampling intensively in cyphelloid groups, and developing multi-gene datasets for cyphelloid fungi and related taxa. Other workers with interests in cyphelloid taxa include Dr. R. G. Thorn (University of Western Ontario), Dr. S. A. Redhead (Agriculture Canada), and Dr. R. Agerer (University of München). Our work complements, but does not duplicate the research of these other workers. As far as we are aware, there are no other research groups studying molecular systematics of aquatic homobasidiomycetes.

**Significance**

The proposed research will increase awareness of a fascinating and controversial group of fungi. Sequence data, cultures, and herbarium materials produced in this research will provide resources for other mycological studies, as well as baseline information about biodiversity. The project will provide training for a post-doctoral fellow, graduate student, high school students, and high school biology teachers. It will promote international scientific exchange and foster ties between Clark University and its neighborhood schools.

**Timetable**

The project will be conducted over a 36 month period, from September, 2002, to August, 2005 (our current NSF support ends in August, 2002). Collecting, culturing, DNA isolation, and morphological studies will be concentrated during the first year of the project (see "Research plan" for schedule of fieldwork). Molecular studies and preparation of manuscripts will be ongoing, but will be concentrated in the second and third years of the project. We plan to generate and analyze nuc-lsu rDNA of all collections in the second year of the project, and assemble and analyze the four-region rDNA dataset in the third year of the project. Training of high school students and high school teachers will take place in each year of the project (summers).

**Preliminary results**

We have obtained 15 collections and 42 cultures of cyphelloid and aquatic homobasidiomycetes, and related taxa (Table 1), including a culture of the cyphelloid, marine species, *Calathella mangrovii*, which we isolated from a fresh fruiting body (generously provided by Dr. E. B. G. Jones). From these, we have generated 26 nuc-lsu, 5 nuc-ssu, 10 mt-lsu, and 14 mt-ssu rDNA sequences.

***Analyses of nuc-lsu rDNA:*** We aligned 23 new nuc-lsu rDNA sequences to 38 sequences from Moncalvo et al (125, 126), 11 sequences from Langer (108), and 10 sequences from our previous work (36, 82, 180). Equally weighted parsimony analysis resulted in two trees (Fig. 2; see caption for tree statistics). The "backbone" of the tree is weakly supported by bootstrapping, and several major clades sensu Hibbett and Thorn (86) are not resolved, which indicates that the nuc-lsu rDNA is not ideal for inferring higher-level phylogenetic relationships in homobasidiomycetes (Fig. 2). Nevertheless, 28 nodes receive at least 70% bootstrap support (Fig. 2). Cyphelloid and aquatic homobasidiomycetes are polyphyletic, as expected, but several clusters of taxa are resolved. One particularly noteworthy clade includes the marine gasteromycete, *Nia vibrissa*, which is strongly supported as the sister group of the mangrove-inhabiting cyphelloid fungus, *Halocyphina villosa*. Also in this clade are another mangrove-inhabiting cyphelloid fungus, *Calathella mangrovii*, several purely terrestrial cyphelloid forms (*Favolaschia pezizoidea*, *Merismodes anomala, Flagelloscypha minutissima*, etc), and the corticioid *Dendrothele acerina* (Fig. 2). These results suggest that *Nia vibrissa*, with its appendaged

spores (Fig. 1b), was derived from terrestrial cyphelloid fungi, via mangrove-inhabiting intermediates.

Space limitations preclude a thorough discussion of the phylogenetic implications of the nuc-lsu rDNA tree, which include the following: 1) the genera *Lachnella, Gloiocephala, Physalacria, Favolaschia,* and *Auriculariopsis* are polyphyletic (*Henningsomyces* may also be polyphyletic, if our isolate of *H. candidus* is correctly identified; however, it may actually be a *Rectipilus*); 2) *Cyphella digitalis* (euagarics clade) is not closely related to *Aleurodiscus* (russuloid clade), contra Donk (59); 3) *Schizophyllum spp.*, *Auriculariopsis ampla, Lachnella villosa*, and *Physalacria bambusae* form a monophyletic group; 4) *Stigmatolemma*, is closely related to *Resupinatus*; 5) *Porodisculus* is nested in *Fistulina*; 6) there have been at least three independent transitions to the marine environment (1, *Nia-Halocyphina*; 2, *Calathella*; 3, *Physalacria maipoensis*) and one independent transition to freshwater (*Limnoperdon*). Thus, although nuc-lsu rDNA does not provide robust resolution of the deeper nodes, it does resolve many of the terminal clades that include cyphelloid and aquatic homobasidiomycetes.

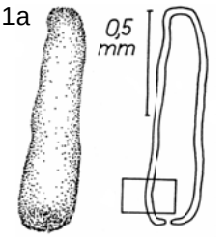
***Analyses of the four-region dataset:*** To assess the improvements in resolution and robustness that may be afforded by a four-region dataset, we analyzed parallel four-region and nuc-lsu rDNA datasets, including 60 new sequences (not all from cyphelloid and aquatic taxa). Six species in the four-region dataset lack nuc-ssu rDNA, two lack mt-lsu rDNA, and two lack both nuc-ssu and mt-lsu rDNA, but the remaining 35 species have sequences from all four regions. The strict consensus tree from the four-region analysis is more resolved than that from the nuc-lsu rDNA analysis, and 26 nodes received bootstrap support of at least 70% in the four-region analysis, compared to only eight nodes in the nuc-lsu rDNA analysis (Fig. 3). All of the eight major clades of homobasidiomycetes sensu Hibbett and Thorn (86) were resolved in the four-region analysis, whereas the euagarics clade, bolete clade, polyporoid clade, and gomphoid-phalloid clade were not resolved in the nuc-lsu rDNA analysis (Fig. 3).

The four-region dataset also provided improved support for certain terminal groupings, including the focal groups of the proposed research. Specifically, the four-region dataset provided strong (94%) bootstrap support for the *Schizophyllum-Fistulina* clade (which also includes *Lachnella villosa*, *Physalacria bambusae*, and *Auriculariopsis ampla*), whereas the nuc-lsu rDNA dataset provided only weak (49%) bootstrap support for this group (Fig. 3). The four-region dataset also provided moderately strong (81%) bootstrap support for the *Nia-Henningsomyces* clade (which also includes *Favolaschia pezizoidea, Halocyphina villosa*, and *Merismodes anomala*), whereas the nuc-lsu rDNA dataset did not support monophyly of this group (because *Henningsomyces candidus* was placed as the sister group of *Pluteus*; Fig. 3). Finally, the four-region dataset provided strong (79-100%) bootstrap support for the placements of four species outside of the euagarics clade that may be related to cyphelloid and aquatic homobasidiomycetes (Fig. 3a), including *Auriculariopsis (Phlebia) albomellea* (130), *Physalacria inflata,* and two species of the *Sistotrema*, which appears to be polyphyletic. The placements of three of these taxa agree in the nuc-lsu rDNA analysis, but they are not strongly supported (Fig. 3b).

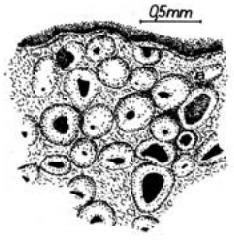
In summary, preliminary results confirm the view that cyphelloid and aquatic homobasidiomycetes are polyphyletic, and resolve many clusters of related taxa. Many cyphelloid forms occur in the *Schizophyllum-Fistulina* clade and the *Nia-Henningsomyces* clade, which also includes three marine species, and there are probably many more cyphelloid species in these clades that have yet to be sampled.

**Fig. 1.** Fruiting bodies (**Fig. 1a**) and spores (**Fig. 1b**) of terrestrial cyphelloid taxa (*Henningsomyces candidus*, *Stigmatolemma poriaeforme*), ballistosporic, marine, cyphelloid taxa (*Halocyphina villosa*), and statismosporic, marine taxa (*Digitatispora marina, Nia vibrissa*).
**Fig. 2.** Phylogenetic relationships of cyphelloid and aquatic homobasidiomycetes inferred from nuc-lsu rDNA (strict consensus of two trees, 2480 steps, CI=0.295, RI=0.585). Taxa in bold include cyphelloid (C) and aquatic (A) species, and putatively related taxa. Names in quotation marks represent questionable identifications or taxonomically controversial names. For explanation of other symbols, see the figure.
**Fig. 3.** Phylogenetic relationships of cyphelloid and aquatic homobasidiomycetes inferred from a four-region vs. nuc-lsu rDNA dataset. **Fig. 3a**. strict consensus of 7 trees inferred from four-region dataset (4899 steps, CI=0.378, RI=0.420). **Fig. 3b**. strict consensus of 16 trees inferred from nuc-lsu rDNA (1736 steps, CI=0.365, RI=0.452). Symbols as in Fig. 2.

1a

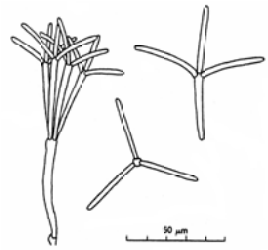



*Henningsomyces candidus*
(Agerer, 1973, fig. 7)

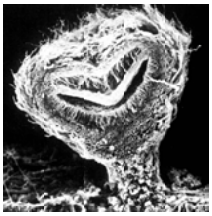



*Stigmatolemma poriaeforme*
(Agerer, 1978, fig. 15a)

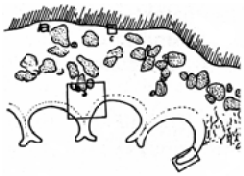
1b



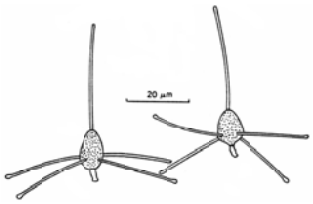

*Digitatispora marina*
(Ingold, 1971, fig. 16.8)



*Halocyphina villosa*
(Nakagiri and Ito, 1991, fig. 9)

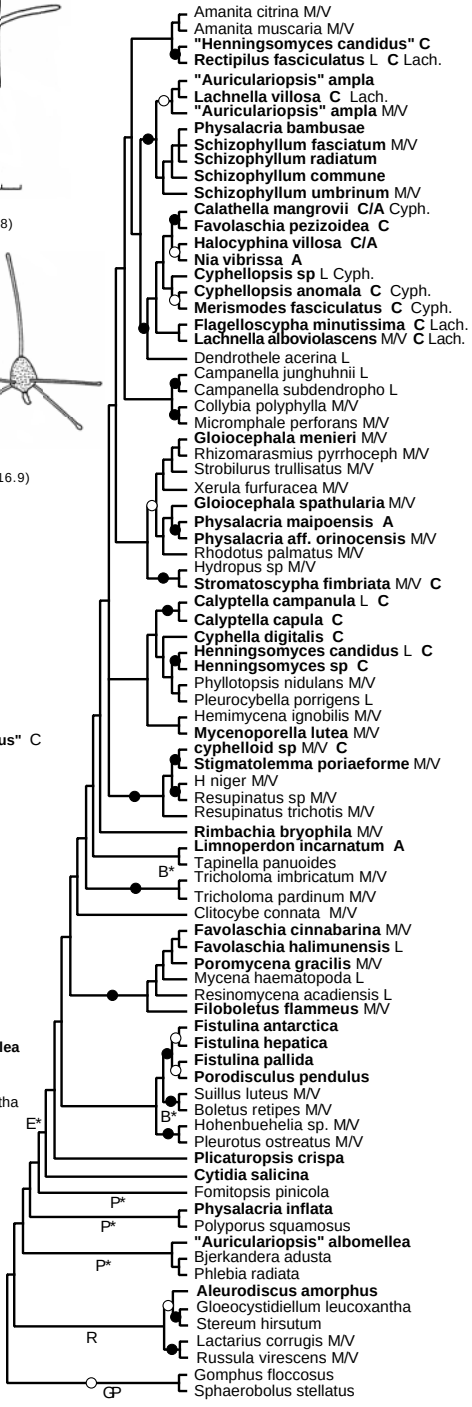


*Stigmatolemma poriaeforme*
(Agerer, 1978, fig. 15b)

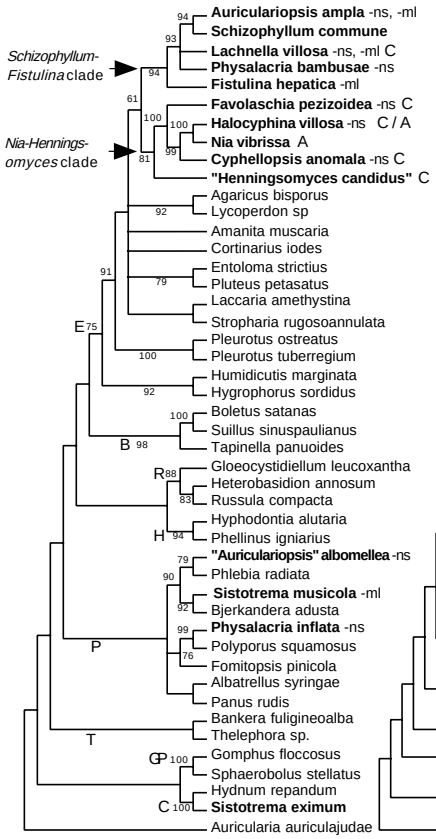



*Niavibrissa*
(Ingold, 1971, fig. 16.9)

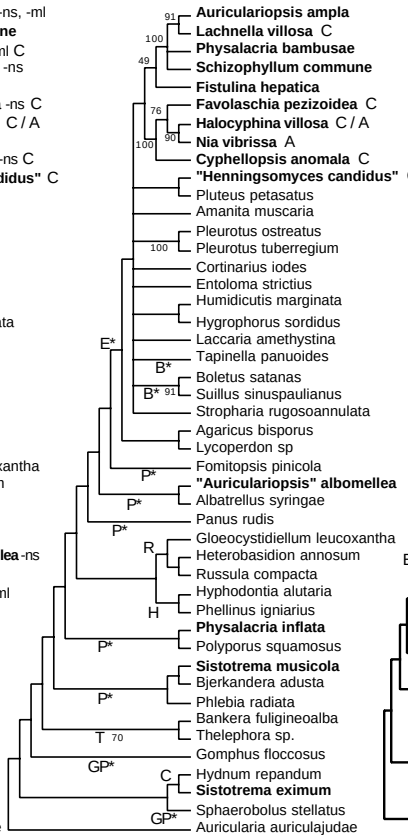
2 nuc-lsu rDNA, strict/2 trees




3a nuc/mt-ssu/lsu rDNA strict/7 trees




3b nuc-lsu rDNA strict/16 trees




Explanation of symbols: C = cyphelloid; A = aquatic; -ns = no nuc-ssu rDNA; -ml = no mt-lsu rDNA
M/V = sequence data from Moncalvo et al. (2000, and unpubl.); L = sequence data from Langer (2001)
● = bootstrap ≥ 90%; ○ = bootstrap 70-89%; other bootstrap values (≥70%) shown along branches
E = euagarics clade; E* = euagarics clade-non-monophyletic; P = polyporoid clade; R = russuloid clade; etc.
Lach. = Lachnellaceae sensu Agerer (1983b); Cyph. = Cyphellopsidaceae sensu Agerer (1983b)